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PHYTO-STIMULATORY ACTIVITY OF SOME FUNGAL BIO-AGENTS ON THE GROWTH OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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ABSTRACT

While screening an array of beneficial microbes, some isolates of *Trichoderma harzianum* and *Chaetomium globosum* showed their promising effect in enhancing seed germination and seedling vigour of sunflower. Higher percentage of germination (94%) was observed in seeds treated with *Chaetomium globosum* compared to 68 % in control. *Trichoderma harzianum* isolates had almost same stimulatory effect on seed germination, root, shoot growth and vigor index. The seeds treated with culture filtrate of *T. harzianum* and *C. globosum*, significantly enhanced the seed germination by 88 and 90%, compared to their respective control. As the consequence, the survivability of seedlings was more; the plants were tall, vigorous and more robust. The treatment also increased the lamina area, number of flowers and head diameter, which were healthy and uniform among replicates The proportion of plants that attained flowering was higher in the treated set (64 and 74%) compared to that of control (25 to 30 %), the length and width of leaves and the diameter of the heads were also found significantly increased in the treated plants. The incidence of *Aspergillus flavus* and *A. niger* was also found depleted in the seeds, treated with bio-agents. Incidence of the pathogenic fungus, *Macrophomina phaseolina* was reduced from 36 to 4 % and 41 to 6 % respectively, in the treated seeds. Incidence of *Alternaria* species was also reduced significantly in the bio-agents culture filtrate treatment.The average lipoxygenase activity of seven-day-old sunflower seedlings was 0.56 and 0.61 for control sets C1 and C2 and 1.01 and 0.94 for *T. harzianum* and *C. globosum* treatments, respectively. The results indicated that there was about 71 and 59 per cent increase in the enzyme activity in *T. harzianum* and *C. globosum* treated seedlings of sunflower, compared to their respective control.

KEYWORDS: Sunflower, Seeds, Seedlings, Fungal Bio-Agents, Vigor and Lipoxygenase Activity

INTRODUCTION

Many microorganisms have plant growth promoting ability. The most extensively studied microorganisms are *Trichoderma* spp., *Corinothyrium minitans, Gliocladium* spp., *Sporodesmium sclerotiorum, Bacillus subtilis* , *Pseudomonos fluorescens, Streptomyces griseoviridis* and yeasts (*Cryptococcus* and *Sporobolomyces*). Some of these organisms also proved in controlling plant diseases (Dandur and Knudsen, 1993). Production of numerous growth promoting factors by *Trichoderma* spp and their contribution to yield enhancement and greater seedling emergence in several vegetable and horticultural crops have been reported (Chang *et al*., 1986; Sivan *et al.*, 1984; Windhan *et al.*, 1986., Lynch *et al.*, 1991). A rapid rooting of *Chrysanthemum* and carnation was observed on treatment with *Trichoderma* spp (Paulitz *et al.*, 1985). Nagarathna *et al.*, (1992) reported an increased activity of Lipoxygenase (LOX) associated with seed germination which were found to exhibit defense mechanism in several plants. Based on these, the present study was undertaken to evaluate the efficacy of five isolates of *Trichoderma harzinum* and one isolate of *Chaetomium globosum* on seed germination and seedling vigor of sunflower *in vitro* and in field conditions.

MATERIALS AND METHODS

Seed Samples: Seed samples of sunflower var. Morden kept for showing were collected from farmers, the moisture content of the seed sample was estimated to be 8 percent and stored in cloth lined bags at room temperature (28 ± 2^0C) for further usage for showing in the field.

Microorganisms: The soil isolates of fungi such as *T. harzianum* (Ti1, Ti2, Ti3, Ti4 and Ti5) and *Chaetomium globosum* were obtained from the stock culture collection of Department of Studies in Biotechnology, University of Mysore, Mysore. The fungi were grown on Potato Dextrose Agar (PDA) plates for a week under alternate cycles of 12/12h Near Ultra Violet (NUV) light and darkness at 22 ± 2^0C.

Seed Treatment: Sunflower seeds (400) were coated with two percent (w/v) gum arabic as an adhesive, air dried till they become sticky. The seeds were then rolled on 8^{th} day old culture of T. *harzianum* and the spore load was measured as 4.5x105 spores/g of seeds, using a haemocytometer.

Fungal Culture Filtrate Treatment: *T. harzianum* and *C. globosum* isolates which were found to enhance the seed germination was cultured on Potato Dextrose Broth (PDB) of pH 6.5 in a series of 500 ml Erlenmeyer flasks containing 150 ml of the medium. For this purpose, the flasks were inoculated with the culture discs of 5mm diameter, obtained from the margin of 8^{th} day old actively cultures growing on PDA. The flasks were incubated for 15 days under alternate cycles of 12/12h, NUV and darkness, at 22 ± 2^0 C. The culture filtrate of each fungus was separately filtered through Whatman No.1 filter paper followed by milipore septa (pore size 0.45µm) and used to treat the seeds. 400 seeds were soaked for 24h at 22 ± 2^0 C. The soaked seeds were then air dried and used for evaluating mycoflora, seed germination and seedling growth.

Seed Mycoflora: To assess the incidence of seed mycoflora, the soaked seeds were plated equidistantly on moist blotter discs in the perspex plates. Such plates were incubated under 12 /12h alternate cycles of NUV/ light darkness and at 22 ± 2^0 C for seven days (Anonymous, 1999). On the eight day, the seeds were evaluated for the incidence of seed mycoflora with the aid of stereo-binocular and compound microscopes.

Evaluation of Germination of Treated Seeds: Treated sunflower seeds were placed between wet blotter sheets at the rate of 100 seeds/ replicate in four replicates. The seeds in the paper towels were incubated according to the standard procedures (Anonymous, 1999). The percent seed germination was recorded and the vigor index of the seedlings was calculated using the formula of Abdual Baki and Anderson (1973).

Soil Amendment Studies: Among the fungi used, Ti3 isolate of *T. harzianum* and *C. globosum* were considered for the soil amendment based on their *in vitro* performance. Both the fungi were separately cultured in polyethylene bags containing sterilized coffee husk added with wheat bran (1: 1) having 18-20% moisture content for 15 days. The cultures with coffee husk was mixed well and broadcasted to the experimental plot at the rate of 1000g/m^2 area and covered with 2cm moist soil. The inoculated soil was incubated for a period from 4 to5 days to facilitate establishment of the fungi.

Field Performance: The experiment was carried out during Kharif 2008 and 2009 in randomized block design, in four replicates. Soil not amended with fungi served as control. In each row, 100 seeds (10 cm between seeds) were sown in moistened soil bed and allowed to grow with sufficient irrigation. Growth promotion and yield parameters were determined periodically, after emergence. During the course of experiment, no herbicides or fertilizers were applied at any stage of the plant growth and weeding was done manually, every fortnight. The data from the experiments were compiled and subjected to statistical analysis (DMRT).

LOX Enzyme Assay: The seeds were surface sterilized with 2%(v/v) sodium hypochlorite solution for 3 min, washed in sterilized distilled water and soaked in 12 day old culture filtrates of *T. harzianum* and *C. globosum* for 24 h. The treated seeds were then air dried and transferred to moist blotters and incubated at 22 ± 2^0C. Seeds soaked in sterile distilled water and PDB maintained under similar conditions were served as controls.

Extraction of Enzyme: 0.5g shoot tips of sprouted seeds were macerated to a fine paste with 0.5g acid washed sand in a mortar at 4^0C. 3ml of cold, 0.2M sodium phosphate buffer (pH 6.5) was used for extraction. The homogenate was centrifuged at 14,000 rpm for 60 min at 4^0 C. The supernatant was used as the enzyme source.

Preparation of Substrate: Substrate for LOX assay was prepared according to the method described by Axelord *et al.* (1981). Enzyme activity was measured by following the procedure of Borthakur *et al.* (1987). The activity was determined spectro photometrically by monitoring the appearance of conjugated diene hydroperoxide at 234nm. The reaction mixture contained 2.7 ml of sodium phosphate buffer (0.2M, pH.6.5) and 0.3 ml of substrate, whose reaction was initiated by adding the enzyme extract (50µl) and the change in absorbance was recorded for 3min using a Hitachi U-2000 spectrophotometer. The enzyme activity was expressed as change in the absorbance mg^{-1} protein min^{-1}. The protein content of each extract was estimated following the procedures of Lowry *et al.* (1951), using BSA (Sigma, USA) as standard. Each experiment was repeated thrice and the data were subjected to statistical analysis. The effects of treatment were compared by mean separation procedure at 5% level, considering the percentage values after Arcsine transformation.

RESULTS

Effect of Seed Treatment with Fungal Isolates

All the fungal isolates enhanced the seed germination and seedling vigor over control. The percentage of germination was significantly high in seeds treated with fungal cultures (94 %) when compared to 68% in control (Table 1). Higher percentage of germination (94%) was observed in seeds treated with *C. globosum*. *T. harzianum* isolates had almost same effect on seed germination, root/shoot growth and vigor index. The fungal seed treatment also increased the mean root/shoot length of resulting seedlings. However, the shoot length of seedlings obtained from C. *globosum* treated seeds was shorter (7.5cm). Among *T. harzianum* isolates, Ti3 was very promising in enhancing the overall seedling vigor.

Effect of Culture Filtrate Seed Treatment with *T. harzianum* (Ti3) and *C. globosum*

The seeds treated with the culture filtrate of *T. harzianum* (Ti3) and *C. globosum* showed significantly enhanced the seed germination to 88 and 90%, respectively, compared to control (Table 2). Culture filtrate treatments also increased the root and shoot length of the seedlings. The vigor index of the seedlings rose out of *T. harzianum* (Ti3) and *C. globosum* culture filtrate treatment was significantly high i.e. 2473 and 2569.

Field Evaluation

- **Effect of Seed Inoculation and Culture Filtrate Treatment on Field Emergence and Other Agronomic Characters of Sunflower Plants**

Seed treatment with cultures and culture filtrates revealed the significantly increased field emergence (Table 3 and 4). The field emergence was found between 51 and 62 % in seeds treated with the cultures of the fungal isolates compared to 15 % and 25 % in control sets. As the consequences of treatment, plants were tall, robust and more vigorous. The survivalibility of seedlings was more in *T. harzianum* (Ti3) and *C. globosum* treated seeds. The treatment also increased the leaf area and flower head diameter, which were healthy and uniform.

- **Effect of Soil Application of Fungal Isolates on Field Performance of Sunflower**

The amendment of soil with selected fungal isolates significantly increased the emergence and survival of the seedlings (Table 5). The plants obtained from treated seeds were taller than those of the untreated ones. The proportion of plants that attained flowering was higher in treated set (64 % and 74%) when compared to control (25 % and 30%), the length and width of leaf and diameter of the head were also significantly increased in the treated plants.

Seed Mycoflora: The incidence of seed-borne fungi except *Cladosporium cladosporioides* were comparatively lower on seeds treated with culture filtrates of antagonists (Table 6) compared to that of untreated seeds. *Aspergillus flavus* and *A. niger* incidence was also found lower in the treated seeds. Incidence of pathogenic fungus, Macrophomina *phaseolina* was reduced from 36 % and 41 % in control to 4 % and 6% in treated seeds. Incidence of *Alternaria* spp. was also reduced significantly in culture filtrate treatment.

Effect of Seed Treatment on Lipoxygenase Activity in Sunflower

The enzyme activity in untreated sunflower (C1) ranged from 0.03 ~ 1.90mg^{-1} protein min^{-1} during 7 days of incubation. In seedlings treated with PDB (C2), the enzyme activity ranged from 0.27~ 2.21 during the same period of time. In *T. harzianum* (Ti3) treated seedlings, the enzyme activity was in the range of 0.43 ~ 2.69. Similarly, *C. globosum* treated seedlings showed the enzyme activity ranging from 0.23 ~ 2.72 mg protein min^{-1} during the same period of incubation. The average enzyme activity of seven day- old seedlings of sunflower was 0.56 and 0.61 for control sets C1and C2, 1.01 and 0.94 for *T. harzianum* and *C. globosum* treatments. To calculate the percent increase in the enzyme activity in treatments over untreated ones, the means of the controls were considered. This showed that there was about 71 and 59 percent increase in the enzyme activity in *T. harzianum* and *C. globosum* treated seedlings of sunflower, respectively over the control (Figure 1) .

DISCUSSIONS

Several fungi are known to act as a stimulant of plant growth. Certain sterile and sporulating fungi from Zoysia grass enhanced the growth remarkably in a variety of crops at and beyond the seedlings stages (Hyakumachi *et al.,* 1993a; Shivanna *et al*., 1994). In the present investigation, different isolates of *T. harzianum* showed varied effect on sunflower growth. This variation is most probably due to the variation in the quality and quantity of phyto-stimulatory compounds. It also depends on the varied level of penetration and establishment in the host system, which in turn, triggers the synthesis of enzymes/hormones ie., necessary for the plant growth. The most potent isolates trigger the synthesis of phyto stimulant at an early stage resulting in the development of health and robust root system. Due to this, plant further take up more nutrients from the soil, establish well and give better yield. A few isolates of *T. harzianum* are reported to be highly potential in enhancing the plant growth (Windham *et al.,* 1986; Lynch *et al.,* 1991). In the field condition, the plants treated with the fungal spores as well as culture filtrate were very much stimulated. The triggering of root growth of different crops by fungal metabolites is reported (Kimura *et al*., 1993; Hyakumachi *et al.,* 1993b).

The leaves were found larger in fungi treated plants. Once there is a stimulated growth, it is quite possible for the easy establishment of leaves. Growth enhancement of a variety of crops by sterile fungal isolates is usually determined at four weeks (Dewan and Sivasitharam, 1989; Hyakumachi *et al.,* 1993a; Shankar *et al.,* 1993). However, reports are available on the growth promotion caused by non sterile fungi such as *Rhizoctonia solani* and species of *Trichoderma,* upto and including seed stage (Sneh *et al*., 1986; Windham *et al.,* 1986) . In the present study, the fungal isolates promoted growth and yield of the crop. *T. harzianum* (Ti3) and *C. globosum* were effective both *in vitro* and *in vivo* in enhancing the overall growth. Although *T. harzianum* (Ti3) enhanced growth, in the present study, it was less effective with respect to

C. globosum isolate in increasing the yield. Many *Trichoderma* isolates have reported to enhance the growth of bent grass, cucumber, ryegrass, tomato, bean, radish and chilli plants (Hyakumachi *et al.,* 1993a; Kleifield and Chet, 1992).

The antagonistic action of *T. harzianum* (Ti3) and *C. globosum* against phytopathogenic fungi is attributed to the secretion of cell wall hydrolytic enzyme or to the production of antibiotics. According to Schirmbock *et al*. (1994) some antibiotics and hydrolytic enzymes co-operated synergistically in antagonism. The reduced incidence of seed mycoflora due to culture filtrate treatment can be attributed to antifungal compounds, which are involved in the defense mechanism of the plants. Several reports are available on the antifungal properties of some fungal cultural filtrates (Elad *et al.,* 1982., Chand and Logan 1984; Beagle - Ristiano and Papavizas, 1985). Some fungi produce both stimulatory and inhibitory compounds (Culter and Jacyno 1991; Daliya and Tewari 1991). This is the most probable reason for the uneven expression of fungi as well as the plant growth with the treatment.

The combined delivery system of microbes with seed is recommended by many workers (Baker *et al* 1986; Chang *et al.,* 1986; Lynch *et al.,* 1991). The use of potential microbes in solid matrix either with peat or with clay or with allied products are in practice to get better yield of the crops. In this study, the soil was amended with substrate colonized by the fungus. The growth of *T. harzianum* on the coffee husk + wheat bran was poor compared to *C. globosum* on the same. This is because *C. globosum* is a well known cellulolytic fungus, that can easily degrade and utilize the ingredients and hence, it establishes and flourishes well. On the other hand, the substrate after digestion may also serve as fertilizer that might promote the activities of other soil borne saprophytic organisms. This might also be one of the reasons for the luxuriant growth of the plants in the husks + wheat bran amended soil. The stimulation of plant growth is most probably due to the production of hormones like auxins.

The fungi alone produce enzymes into the host system for increased synthesis of hormones or allied compounds. It has been observed that the compounds like jasmonate, fusicoccin, amidenin, altechromones A and B, spicifernin sescandelin dihydroampullicin are produced by several fungi that are involved in phytostimulation (Kimura *et al.,* 1993). The investigators have noticed that one of the important turning points that triggered stimulation was due to the production of LOX enzyme in fungal culture filtrate treatment of fungi to sunflower seeds. It is known that the enzyme LOX plays an important role in the seed germination and defense mechanism in plants (Gross and Parthier, 1994). The *Lasiodiplodia theobromae* which produces the classical hormone jasmonate triggers the activity of LOX (Bell and Mullet, 1991; Grimes *et al.,*1992). The mechanism of growth enhancement by inoculation with *T. harzianum* and C. *globosum* is not well understood. Cowan (1979) suggested that some growth promoting isolates of *Phialophora graminicola* increase the mineral nutrient uptake by plants in the same way as do mycorrhizal fungi.

The results of the present investigation indicate that *T. harzianum* (Ti3) and *C. globosum* isolates are the determinant factors for the significant enhancement of sunflower plant growth. More work is needed with regard to the efficiency of different isolates in the field year to year, their continued and prolonged survivability, and the impact of other microbes and the response of isolates to varied climatic factors as well.

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APPENDICES

Table 1: Effect of Fungal Culture Treatment on Seed Germination and Seedling Vigour of Sunflower

Fungal Isolates (Bio-Agents)	Seed Germination (%)	Mean Root Length (cm)	Mean Shoot Length (cm)	Vigour Index (VI)
Control 1	70d	9.0c	10.0bc	1291cdef
Control 2	70d	9.0c	11.0b	1398cde
Trichoderma harzianum (Ti1)	90bc	11.0bc	10.0b	1803bcd
T. harzianum (Ti2)	80c	10.0bc	11.0b	1617cd
T. harzianum (Ti3)	90b	14.0a	15.0a	2529a
T. harzianum (Ti4)	90b	12.0d	12.0b	2192a
T. harzianum (Ti5)	90b	12.0b	10.0b	1968bc
Chaetomium globosoum	90a	15.0a	8.0c	2104b

Figures followed by same letter (s) are not significantly different at 5% level when subjected to Duncan Multiple Range Test (DMRT)

Table 2: Effect of Culture Filtrate of *Trichoderma harzianum* and *Chaetomium globosoum* on Seed Germination and Seedling Vigour of Sunflower

Fungal Isolates (Bio-Agents)	Seed Germination (%)	Mean Root Length (cm)	Mean Shoot Length (cm)	Vigour Index (VI)
Control 1	70.0b	9.0c	12.0bc	1.373bcd
Control 2	70.0b	9.0c	11.0bc	1294bc
Trichoderma harzianum (Ti3)	90.0a	13.0b	15.0a	2473b
Chaetomium globosoum	90.0a	15.0a	14.0b	2569a

Figures followed by same letter (s) are not significantly different at 5% level when subjected to DMRT.

Table 3: Influence of *Trichoderma harzianum* and *Chaetomium globosum* on Field Emergence and Other Growing on Parameters of Sunflower

Treatments (Bio Agents)	Plant Growth Parameters						
	A	B	C	D	E	F	G
C1	70.0a	6.0a	100.0a	13.0a	9.0a	70.0a	7.0a
C2	70.0a	6.0b	90.0a	8.0ab	4.0d	50.0b	4.0c
Ti1	60.0c	5.0b	90.0a	7.0b	5.0c	40.0c	4.0c
Ti2	70.0ab	5.0c	100.0b	11.0bc	6.0b	60.0b	6.0b
Ti3	60.0c	4.0c	90.0b	100.0cd	5.0c	3.0c	4.0d
Ti4	60.0ab	5.0cd	90.0b	10.0d	5.0c	3.0c	4.0d
Ti5	5.0d	4.0cd	80.0bc	8.0d	4.0d	30.0c	3.0d
Cg	50.0d	4.0d	90.0c	8.0d	4.0d	30.0c	3.0d

Figures followed by same letter (s) are not significantly different at 5% level when subjected to DMRT.

A. Percent filed emergence after 7 days of sowing	E. Lamina width (cm)
B. Height of the plant above ground level (cm)	F. Percent of plants attained flowering after 30 days of sowing
C. Percent surviving after 30 days of sowing	G. Diameter of head (cm)
D. Lamina length (cm)	Cg. *Chaetomium globosum*

Table 4: Field Evaluation of Sunflower Treated with the Culture Filtrate of *Trichoderma harzianum* and *Chaetomium globosum*

Treatments (Bioagents)	Plant Growth Parameters							
	A	B	C	D	E	F	G	H
C1	20.0d	50.0c	8.0c	90.0b	20.0c	8.0c	4.0b	3.0c
C2	30.0c	40.0c	8.0c	90.0b	30.0c	8.0c	4.0b	4.0c
Ti3	50.0b	70.0b	12.0b	90.0a	60.0b	11.0b	7.0a	6.0d
Cg	60.0a	80.0a	14.0a	100.0a	70.0a	14.0a	9.0a	7.0a

Figures followed by same letter (s) are not significantly different at 5% level when subjected to DMRT.

A. Percent of early emergence (after 4 days)
B. Percentage of total emergence (after 7 days)
C. Height of the plant above ground level (cm)
D. Percent surviving (after 30 days)
E. Percent of plants attained flowering (after 30 days)
F. Length of lamina (after 30 days)
G. Width of lamina (after 30 days)
H. Diameter of head (cm)Cg. *Chaetomium globosum*

Table 5: Field Performance of Sunflower in Soil Amended with *Trichoderma harzianum* and *Chaetomium globosum*

Treatments	Growth Parameters								
	A	B	C	D	E	F	G	H	I
C1	20.0b	30.0b	7.0b	12.0b	90.0b	30.0b	8.0b	4.0b	4.0b
C2	20.0b	30.0b	8.0b	13.0b	90.0b	30.0b	8.0b	4.0b	4.0b
Ti3	70.0a	80.0a	9.0a	17.0ab	90.0a	60.0a	12.0a	7.0a	7.0a
Cg	70.0a	80.0a	11.0a	20.0a	100.0a	70.0a	14.0a	8.0a	8.0a

Figures followed by same letter (s) are not significantly different at 5% level when subjected to DMRT.

A. Percent of early emergence (after 4 days)
B. Total Percent filed emergence
C. Height of the plant above ground level (after 7 days)
D. Height of the plant above ground level (after 15 days)
E. Percent surviving (after 30 days)
F. Length of lamina (after 30 days)
G. Width of lamina (after 30 days)
H. Diameter of head Cg. *Chaetomium globosum*

Table 6: Effect of Culture Filtrate of *Trichoderma harzianum* and *Chaetomium globosum* on Seed Mycoflora

Mycoflora	% Disease Incidence			
	A	B	C	D
Aspergillus flavus	30.0b	60.0a	05c	03c
A. flavus-columnaris	06	06	03	01
A. niger	30.0b	70.0a	10.0c	01d
Alternata alternata	10.0a	9.0b	01c	03c
A. tenuissima	04	06	-	-
Cladosporium cladosporioides	40.0a	10.0c	30.0b	40.0a
Fusarium moniliforme	20.0a	10.0b	06c	06c
F. solani	40.0a	30.0b	10.0c	09c
F. oxysporum	10.0a	10.0a	4.0b	03b
Macrophomina phaseolina	30.0a	40.0a	04b	05b
Rhizopus spp.	20.0a	20.0a	04b	06b

Figures followed by same letter (s) are not significantly different at 5% level when subjected to DMRT.

A.	Seed + sterile distilled water	C.	Seed + *Trichoderma harzianum*
B.	Seed + gum arabic	D.	Seed + *Chaetomium globosum*

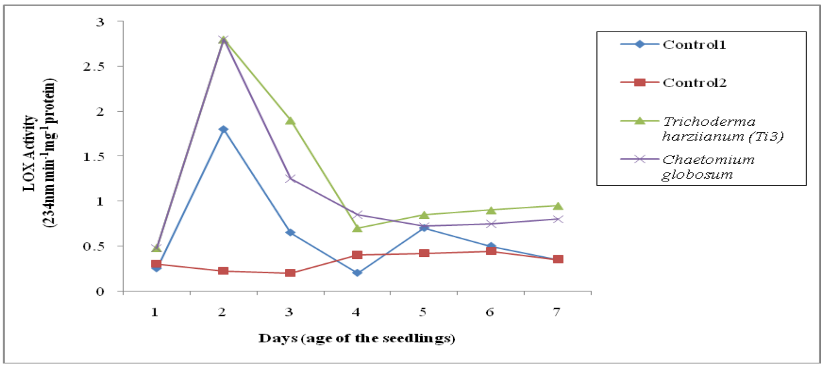

Figure 1: Effect of Seed Treatment on Lipoxygenase Activity in Sunflower